cellsciences.com

Human IL-6 ELISPOT Kit
PVDF Format

Catalog No. CDK131A	**Quantity:** 5 x 96 Tests	With Sterile Plates
Catalog No. CDK131B	**Quantity:** 10 x 96 Tests	With Sterile Plates
Catalog No. CDK131C	**Quantity:** 15 x 96 Tests	With Sterile Plates
Catalog No. CDK131D	**Quantity:** 20 x 96 Tests	With Sterile Plates

1. Intended Use

The ELISPOT assay is a highly specific immunoassay for the analysis of cytokine and other soluble molecule production and secretion from T-cells at a single cell level in conditions closely comparable to the *in-vivo* environment with minimal cell manipulation. This technique is designed to determine the frequency of cytokine producing cells under a given stimulation and the comparison of such frequency against a specific treatment or pathological state. The ELISPOT assay constitutes an ideal tool in the investigation of Th1 / Th2 responses, vaccine development, viral infection monitoring and treatment, oncology, infectious disease, autoimmune diseases, and transplantation.

Utilizing sandwich immuno-enzyme technology, the Cell Sciences® ELISPOT assay can detect both secreted cytokines and single cells that simultaneously produce multiple cytokines. Cell secreted cytokines or soluble molecules are captured by coated antibodies avoiding diffusion in supernatant, protease degradation or binding on soluble membrane receptors. After cell removal, the captured cytokines are revealed by tracer antibodies and appropriate conjugates.

2. Reagents Provided

- 96-well PVDF bottomed plate
- Capture antibody for IL-6 (0.5 ml per vial, supplied sterile).
- Biotinylated detection antibody (lyophilized, resuspend each vial in 0.55 ml).
- Streptavidin - Alkaline Phosphatase conjugate (50 µl per vial).
- Bovine Serum albumin (BSA).
- Ready-to-use BCIP/NBT substrate buffer (25 ml per vial, 2 vials)

Store all reagents between 2 and 8°C.

3. Materials / Reagents required but not provided

- Cell culture media
- Cell stimulation reagents
- CO_2 incubator
- Ethanol
- Tween 20
- Phosphate buffered saline (PBS)

Cell Sciences, Inc.
480 Neponset Street
Building 12A
Canton, MA 02021

Toll Free: 888 769-1246
Phone: 781 828-0610
Fax: 781 828-0542

E-mail: info@cellsciences.com
Web Site: www.cellsciences.com

4. Storage

Store kit reagents between 2 and 8°C. Immediately after use, remaining reagents should be returned to cold storage (2 to 8°C). The expiration date of the kit and reagents is stated on the box front labels. The expiration of the kit components can only be guaranteed if the components are stored properly, and in the case of repeated use of one component, the reagent is not contaminated by the first handling.

5. Safety and Precautions for Use

- **For Research Use Only. Not to be used as a diagnostic test.**
- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures , e.g.CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.
- When not in use, kit components should be stored refrigerated or frozen as indicated on the vial or bottle labels.
- All reagents should be warmed to room temperature before use.
- Cover or cap all reagents when not in use.
- Do not mix or interchange reagents between different lots.
- Do not use reagents beyond the expiration date of the kit.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination.
- Use a clean plastic container to prepare the washing solution.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- **BCIP/NBT buffer** is potentially carcinogenic and should be disposed of appropriately, caution should be taken when handling this reagent, always wear gloves.
- Follow incubation times described in the assay procedure.

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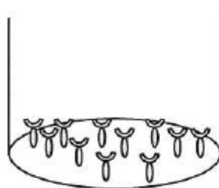
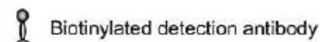
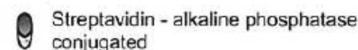
6. Principle of the Method

A capture antibody highly specific for the analyte of interest is coated to the wells of a PVDF bottomed 96 well microtiter plate either during kit manufacture or in the laboratory. The plate is then blocked to minimize any non-antibody dependent unspecific binding and washed. Cell suspension and stimulant are added and the plate incubated allowing the specific antibodies to bind any analytes produced. Cells are then removed by washing prior to the addition of Biotinylated detection antibodies which bind to the previously captured analyte. Enzyme conjugated streptavidin is then added binding to the detection antibodies. Following incubation and washing, substrate is then applied to the wells resulting in “purple” colored spots which can be quantified using appropriate analysis software or manually using a microscope.

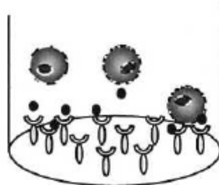
1. 96-PVDF bottomed-well plates are first treated with 35% ethanol and then coated with capture antibody



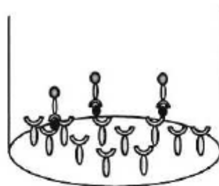




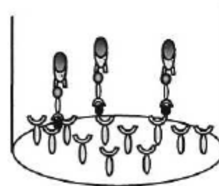
2. Incubation of cells in the coated microwell



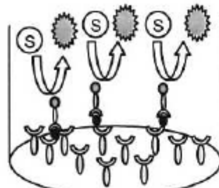
3. Cell removal by washing. Incubation with biotinylated antibody



4. Incubation with streptavidin – alkaline phosphatase conjugated



5. Addition of substrate BCIP/NBT and monitoring of spot formation.



7. Reagent Preparation

Phosphate Buffered Saline (10X Concentrated Solution) (Coating Buffer).

For 1 liter, weigh out: 80 g NaCl + 2 g KH_2PO_4 + 14.4 g $Na_2HPO_4 \bullet 2H_2O$. Add distilled water to 1 liter. Adjust the pH of the solution to 7.4 +/- 0.1 if necessary. **Dilute the solution to 1X before use.**

Cell culture media + 10% serum (Blocking Buffer)

For one plate add 1 ml serum (e.g. FCS) to 9 ml of culture media (use the same cell culture medium as the medium used to derive the cell suspension).

1% BSA PBS Solution (Dilution Buffer)

For one plate, dissolve 0.2 g of BSA in 20 ml of 1X PBS.

0.05% PBS-T Solution (Wash Buffer)

For one plate dissolve 50 µl of Tween 20 in 100 ml of 1X PBS.

35% Ethanol (PVDF Membrane Activation Buffer)

For one plate mix 3.5 ml of ethanol with 6.5 ml of distilled water.

Capture Antibody

This reagent is supplied sterile. Once opened, keep the vial sterile or aliquot and store at -20°C. For optimal performance prepare the Capture Antibody dilution immediately before use.

Dilute 100 µl of capture antibody in 10 ml of 1X PBS and mix well.

Detection Antibody

Reconstitute the lyophilized antibody with 0.55 ml of distilled water. Gently mix the solution and wait until all the lyophilized material is back into solution.

If not used within a short period of time, reconstituted Detection Antibody should be aliquoted and stored at -20°C. In these conditions the reagent is stable for at least one year. For optimal performance prepare the reconstituted antibody dilution immediately prior to use.

Dilute 100 µl of antibody into 10 ml Dilution Buffer and mix well.

Strepatavidin-Alkaline Phosphatase Conjugate

For optimal performance prepare the Streptavidin-AP dilution immediately prior to us.
For 1 plate dilute 10 µl of Streptavidin-AP conjugate into 10 mL Dilution Buffer and mix well.

DO NOT KEEP THE DILUTIONS FOR FURTHER EXPERIMENTS.

8. Sample and Control Preparation

Positive Assay Control: IL-6 production by PBMC upon stimulation by LPS.

This protocol is given as a suggestion.

Dilute PBMC in culture media (e.g. RPMI 1640 supplemented with 2 mM L-glutamine and 10% heat inactivated fetal calf serum) containing 1 µg/ml LPS (Sigma, Saint Louis, MO). Distribute 1 x 10^4 to 2.5 x 10^4 cells in antibody coated PVDF-bottomed-wells and incubate for 15-20 hours in an incubator.

For antigen specific stimulation, the optimal concentration of the antigen and the optimal concentration of number of cells have to be determined experimentally, as it is depending on the frequency of cytokine producing cells.

Negative Assay Control

Dilute PBMC in culture media to give an appropriate cell number (same number of unstimulated cells as stimulated sample cells) per 100 µl with no stimulation.

Sample

Dilute PBMC in culture medium and stimulator of interest (i.e. Sample, Vaccine, Peptide pool or infected cells) to give an appropriate cell number per 100 µl.

Optimal assay performances are observed between 1 x 10^5 and 2.5 x 10^5 cells per 100µl.

Stimulators and incubation times can be varied depending on the frequency of cytokine producing cells and therefore should be optimized by the testing laboratory.

9. Method

Prepare all reagents as shown in section 7 and 8.
Note: For optimal performance prepare the Streptavidin-AP dilution immediately prior to use.

Assay Step		Details
1.	Addition	Add 25µl of 35% ethanol to every well
2.	Incubation	Incubate plate at room temperature (RT) for 30 seconds
3.	Wash	Empty the wells by flicking the plate over a sink & gently tapping on absorbent paper. Thoroughly wash the plate 3x with 100µl of 1X PBS per well
4.	Addition	Add 100µl of diluted **capture antibody** to every well
5.	Incubation	Cover the plate and incubate at 4°C overnight
6.	Wash	Empty the wells as previous and wash the plate once with 100µl of 1X PBS per well
7.	Addition	Add 100µl of **complete media with 10% serum** to every well
8.	Incubation	Cover the plate and incubate at RT for 2 hours
9.	Wash	Empty the wells as previous and thoroughly wash once with 100µl of 1X PBS per well
10.	Addition	Add 100µl of **sample, positive and negative controls** cell suspension to appropriate wells providing the required concentration of cells and stimulant (cells may have been previously stimulated see section 8.)
11.	Incubation	Cover the plate and incubate at 37°C in a CO_2 incubator for an appropriate length of time (15-20 hours). ***Note: do not agitate or move the plate during this incubation***
12.	Addition	Empty the wells and remove excess solution then add 100µl of PBS-T to every well
13.	Incubation	Incubate the plate at 4°C for 10 min
14.	Wash	Empty the wells as previous and wash the plate 3x with 100µl of PBS-T
15.	Addition	Add 100µl of diluted **detection antibody** to every well
16.	Incubation	Cover the plate and incubate at RT for 1 hour 30 min
17.	wash	Empty the wells as previous and wash the plate 3x with 100µl of PBS-T
18.	Addition	Add 100µl of diluted **Streptavidin-AP conjugate** to every well
19.	Incubation	Cover the plate and incubate at RT for 1 hour
20.	Wash	Empty the wells and wash the plate 3x with 100µl of PBS-T
21.	Wash	Peel of the plate bottom and wash both sides of the membrane 3x under running distilled water, once washing complete remove any excess solution by repeated tapping on absorbent paper.
22.	Addition	Add 100µl of ready-to-use **BCIP/NBT buffer** to every well
23.	Development	Incubate the plate for **5-20 min** monitoring spot formation visually throughout the incubation period to assess sufficient colour development
24.	Wash	Empty the wells and rinse both sides of the membrane 3x under running distilled water. Completely remove any excess solution by gentle repeated tapping on absorbent paper

Read Spots: allow the wells to dry and then read results. The frequency of the resulting colored spots corresponding to the cytokine producing cells can be determined using an appropriate ELISPOT reader and analysis software or manually using a microscope.
Note: spots may become sharper after overnight incubation at 4°C.

Plate should be stored at RT away from direct light, but please note color may fade over prolonged periods so read results within 24 hours.

10. Performance Characteristics

Specificity

The assay recognizes natural human IL-6.

To define specificity, several proteins were tested for cross reactivity. There was no cross reactivity observed for any protein tested (IL-1α, IL-1β, IL-10, IL-12, IFNγ, IL-4, TNFα, IL-8 and IL-13).

This testing was performed using the equivalent human IL-6 antibody pair in an ELISA assay.

Reproducibility and Linearity

Intra-assay reproducibility and linearity were evaluated by measuring the spot development following the stimulation (LPS) of 5 different PBMC cell concentrations, 12 repetitions in 1 batch. The data shows the mean spot number, range and CV for the five cell concentrations.

Cells / well	n	Mean number of spots per well	Min	Max	CV%
10000 recommended	12	467	439	533	5.9%
5000	12	340	327	370	3.9%
2500	12	207	190	225	4.7%
1560	12	118	108	129	6.3%
625	12	64	54	76	10.4%

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